# MIXTURES OF HIBISCUS SABDARIFFA STEMS AND ROOTS FOR ANTHOCYANIN SYNTHESIS

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# ABSTRACT

The mixture of *H.sabdariffa* stems and roots were extracted to get anthocyanin. *H.Sabdariffa* plant grows seriously in a warm countries including Malaysia. This plant is usually grown for its calyces and flowers which are mainly used for anthocyanin synthesis. Anthocyanin can also be synthesized from other parts of *H.Sabdariffa* like stems and leaves. The stems of anthocyanin were discarded without any value added product. Thus, this work was done to use the stems and roots mixture for anthocyanin. Besides that, the variations of temperature, time and ethanol-water ratio on the anthocyanin yield were investigated. The anthocyanin was extracted from *H.Sabdariffa* stems and roots waste using ultrasound technique with ethanol-water as solvent. The anthocyanin yield was analysed using High Performance Chromatography (HPLC). The condition where it gave the highest yield of anthocyanin was at contact time of 30 minutes, temperature of 50$^{o}$C and 70:30 of ethanol water solvent. Extraction of anthocyanin from roselle approved the second order kinetic yielding good R2 values of 0.9882 and k values 0.0306.

# ABSTRAK

Campuran batang dan akar tumbuhan Roselle telah diekstrak untuk mendapatkan anthocyanin. *H.sabdariffa* tumbuh di Negara yang mempunyai cuaca yang hangat termasuk Malaysia. Tumbuhan ini biasanya ditanam untuk pucuk dan bunga yang selalu digunakan untuk mendapatkan anthocyanin. Anthocyanin juga boleh di dapati melalui bahagian lain tumbuhan *H.sabdariffa* seperti batang dan juga akarnya di mana batang dan akarnya sering kali dibuang kerana dianggap sebagai bahan buangan. Dengan itu, tujuan projek ini adalah untuk menggunakan campuran batang dan akar untuk mendapatkan anthocyanin. Selain itu, parameter seperti masa, suhu dan nisbah pelarut etanol dan air ke atas hasil anthocyanin telah dikaji. Anthocyanin telah diekstrak daripada batang dan akar *H.sabdariffa* menggunakan teknik ultrasound dimana etanol dan air sebagai pelarut. Hasil anthocyanin telah di analisis menggunakan HPLC. Keadaan di mana ia memberikan hasil anthocyanin yang tertinggi adalah pada masa 30 minit, suhu pada 50$^{o}$C dan pada nisbah pelarut etanol:air 70:30. Penjerapan anthocyanin dari larutan membuktikan pseudo kedua  berhasil dengan nilai R2 ialah 0.9882 dan k nilai 0.0306.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| HPLC | High Performance Liquid Chromatography |
| L | liter |
| mg | milligrams |
| min | minutes |
| ml | milliliter |
| nm | nano metre |
| ppm | Part per million, mg/L |

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# LIST OF SYMBOLS

| | |
|---|---|
| $^{o}C$ | Degree celcius |
| $C_e$ | Equilibrium concentration |
| $C_o$ | Initial concentration |
| $K_f$ | Freundlich constant |
| $K_{1ads}$ | Rate constant of pseudo-first order |
| $K_{2ads}$ | Rate constant of pseudo-second order |
| $R^2$ | Correlation coefficients |
| V | Volume |

# 1 INTRODUCTION

## *1.1 Background of study*

*Hibiscus Sabdariffa* or also known as Roselle is a member of hibiscus family, Malvaceae. This plant has been originated from West Africa but can be found in Malaysia. In Malaysia, roselle is a relatively new crop to create in industry. It was introduced in early 1990s and its commercial planting was first promoted in 1993 by the Department of Agriculture in Terengganu. (**Umi, 2011**). Roselle is an important cash crop grown in the East Coast of Malaysia especially in Terengganu and Kelantan (**Wong et al., 2002**). Roselle is believed to have many useful properties in which different parts of the plant are used for different purposes. In countries like India, Tropical Africa, Philippines and Indonesia, Roselle has been utilised for a long time in producing refreshing beverages, jellies, jams, sauces and preserves (**Esselen and Sammy, 1973; Clydesdale *et al.*, 1979**). As for Malaysia the usage of this product is considered new. The different parts of Roselle are seeds, leaves, stems and calyces and which have been used as vegetables, source of oil, refreshing drinks and food preserves. Other than that, various parts of the Roselle plant have been used in traditional medicine for the prevention of disease such as cardiovascular disease and hypertension. Among parts of Roselle, its fleshy calyces are well-known for its various uses. Its calyces have been suggested as food colorants for food industries, emulsifier for carbonated drinks, jam manufacture, juices and natural food colorants (**Duangmal *et al.*, 2004**).

Beverage that is made from Roselle calyces contains abundant amounts of natural antioxidants such as anthocyanins and vitamin C. Anthocyanins can be found in almost all parts of plant including Roselle. The flowers of *Hibiscus sabdariffa* are rich in anthocyanins (**Cisse *et al.*, 2009**).With various use of anthocyanins, many researchers are attracted to study on anthocyanins of Roselle because of their potential commercial value and it has high possibility to be marketed widely. Recently there has been an increased interest in research on food components such as anthocyanins and other phenolic compounds because of their possible linkage to health benefits including reduction in heart disease and cancer, based on their antioxidant activity (Seeram *et al.*, 2002). With the global

functional food and beverage market expected to reach $109 billion by 2010 (Watkins, 2008), diverse sources of phytochemicals are being explored. Anthocyanins (ACNs) are water-soluble plant pigments responsible for the blue, purple, and red color of many plant tissues (Xianli et al., 2006). As for Roselle,it contains anthocyanin that produces natural red pigments which has been used to make jams jelly, beverages, colouring agent and many more in the food industry. There are a few types of anthocyanin can be found in Roselle. The phenolic content in the plant consists mainly of anthocyanins like delphinidin-3-glucoside, sambubioside, and cyanidin-3- sambubioside mainly contributing to their antioxidant properties (Aurelio *et al*., 2007)

For the extraction of anthocyanins from the mixture of *Hibiscus sabdariffa* stems and leaves, ultrasound technique will be used and analyzation of its content is done by using High Perfomance Liquid Chromatography(HPLC). There are several techniques of extraction can be done which are ultrasound-assisted method, conventional extraction method and microwave-assisted extraction method. Among these, ultrasound-assisted method is the best method for extraction. Previous research for anthocyanins extraction were done but the method used was inefficient. As for ultrasound-assisted method, reasearches on extraction of anthocyanins of *Hibiscus Sabdariffa* calyxes were done. Therefore, for the extraction of anthocyanins from *Hibiscus Sabdariffa* leaves and stems mixture will be made by using ultrasound-assisted method. The reason of using ultrasound method is because of its advantages. The advantages of using ultrasound for food processing, includes: more effective mixing and micro-mixing, faster energy and mass transfer, reduced thermal and concentration gradients, reduced temperature, selective extraction, reduced equipment size, faster response to process extraction control, faster start-up, increased production, and elimination of process steps (Chemat et al., 2011).

The focus of this work is to determine the anthocyanins content from mixture of *Hibiscus Sabdariffa* stems and leaves waste which can give advantages to country to reduce agriculture waste and have more sources in the production of anthocyanins.

## *1.2 Motivation*

In this experiment, anthocyanins will be extracted from the mixture of *H.Sabdariffa* stems and rootss. The research of production of anthocyanins from the mixture of *H.Sabdariffa* stems and roots will be studied in order to use the whole plant of *H.Sabdariffa* instead of its flower. All this time, the only part that are used for production of anthocyanins is the flower, because of that its stems and leaves has been an agricultural waste. This research will help to reduce an agricultural waste, help in improvement of health and also help in improving the production of anthocyanins from *H.Sabdariffa*. With this, Malaysia will be able to enlarge the plantation of *H.Sabdariffa* and has its own production of anthocyanins without need to import from other countries. Therefore, the waste can be treated into wealth.

## *1.3 Problem Statement*

The plantation of Roselle is considered new in Malaysia where only at certain places this plant can be obtained. Therefore the source of anthocyanins is limited and need to be imported from other countries. Other problem is causing an agriculture waste. Many researches have been done before on the production of anthocyanins from Roselle but the researches only focus about production of anthocyanins from flowers. So, people are only interest on flowers that made other parts that were not used like stems and roots were thrown away. So its stems become an agricultural waste since it is not used to extract the anthocyanins. Other than that, the yield separation is low and need to be visible method for the improvement of yield. Therefore this work will be investigated the possibility of stems and leaves mixtures for anthocyanin synthesis using ultrasound-assisted extraction method. With this work, an agricultural waste can be avoided and an alternative way of obtaining anthocyanin is successfully investigated.

## *1.4 Objective*

This research is aims to separate anthocyanins from the mixture of *H.Sabdariffa* stems and roots waste and also to find the kinetic of anthocyanin separation from the mixture of *H.Sabdariffa* stems and roots waste.

## *1.5 Scope of Study*

1.5.1) Extraction of anthocyanins using ultrasound-assisted. During the experiment, observations of the effect of some parameters (time, temperature and solvent ratio) to the separation of anthocyanins from the mixture of H.Sabdariffa stems and roots were done.

1.5.2) Filtration and separation of the sample. The separation process was done by using separation funnel while as for the filtration vacuum filter was used.

1.5.3) Analyzation using High Performance Liquid Chromatography. The content of anthocyanins in the mixture of stems and roots from H.Sabdariffa were detected at the wavelength of 520 and 260 nm.

1.5.4) Kinetic model of separation of anthocyanins from the mixture of H.Sabdariffa stems and roots. The kinetic wasdetermined based on the influence of process parameters that effect the anthocyanins yield.

# 2 LITERATURE REVIEW

## *2.1 Applications of Hibiscus Sabdariffa*

*Hibiscus sabdariffa* or Roselle is known to have many useful properties which different parts of the plant are used for different purposes. Among the useful of this plant is the red and fleshly calyces of the flower are consumed worldwide as a cold beverage or hot drink. These extracts are also used in folk medicine against many complaints that include high blood pressure, liver disease and fever (Wang et al. 2000). As for the leaves and stems of Roselle, some people eat prefer to eat them raw as salads and side dishes. Roselle is also known locally as asam susur, asam paya or Ribena Malaysia, Roselle is used in jams, jellies, sauces and wines. The young leaves and tender stem are eaten raw in salads and chutney. They are also added to curries and some Malaysian dishes as seasoning. The seeds are somewhat bitter but, in Africa, they are ground into meal for human food due to their high protein content. (Yee et al. 2009). Roselle is also used in medical applications for some diseases treatment whether for releasing stress or treatment in deadly disease. For example, in China it is used to treat hypertension, pyrexia, liver damage and leukaemia due to its high content of protocatechuic acid (Tseng et al., 2000). Other than that, roselle is believed to be one of the plants that can help in digestion of human. Studies by Muhammad and Shakib (1995) have shown that roselle can prevent cancer, lower blood pressure and improve the digestive system in humans. Variation applications of roselle can make this plant to be in market and attracts many people.

## *2.2 Anthocyanins in H.sabdariffa stems and roots*

Anthocyanin is the water soluble plant pigments which is responsible for the blue, purple and red colour of many plant tissues. There are a lot of benefits of anthocyanin that make people start to gain interests in it including researchers. Researches were made on the production of anthocyanin from parts of plants. Among the functions of anthocyanin are as pH indicator, food colourant, addition in cosmetics products and many more. Anthocyanin is widely used in food industry, pharmaceutical industry and cosmetic industry which been applied in their products. Anthocyanins from natural sources have become very important for the use of phytochemicals in the preparation of food supplements or

nutraceuticals, functional food ingredients and food additives, and pharmaceutical and cosmetic products (**cisse et al**). Roselle is well known for its rich content of anthocyanin, vitamin C and antioxidants. Anthocyanin can be found in every part of plants including fruits, stems, leaves, roots and flowers including in Roselle. As for Roselle, the anthocyanin from this plant produces natural red pigments. The red pigments are used as food colorants mainly in food industry. Every parts of roselle such as flowers, leaves and stems are believed to have the content of anthocyanin. The phenolic content in the plant consists mainly of anthocyanins like delphinidin-3-glucoside, sambubioside, and cyanidin-3- sambubioside mainly contributing to their antioxidant properties (**Aurelio *et al*., 2007**).

### *2.3 Ultrasound-assisted extraction technique*

Various novel methods including ultrasound-assisted, microwave-assisted and accelerated solvent extraction been introduced for the extraction of nutraceuticals from plants. All of these methods have their own way of extraction but mong these methods of extraction, a few things should be taken into account to get the most efficient of extraction. Extraction process is needed in order to get the anthocyanin from the mixture of stems and leaves of Roselle. The reason of using ultrasound for the anthocyanin extraction is because ultrasound-assisted extraction (UAE) is an inexpensive compare to others method which requires low instruments, simple yet efficient. This technique is easy and efficient and it is less time consuming among other method of extraction. Ultrasound also offers a mechanical effect allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between the solid and liquid phase, and as a result, the solute quickly diffuses from the solid phase to the solvent. (**Wang et al. 2006**). Therefore, time needed for extraction is shorter. Instead, the use of UAE may prevent the possible chemical degradation of targeted compounds due to decreased chemical involvement and reduction in extraction time (Rostagno et al. 2003). Other than that, it was claimed by (Melecchi et al., 2002) that ultrasound-assisted extraction was considered as an efficient method for extraction bioactive compounds from Hibiscus flowers. In this research, solid-liquid extraction process is involved. Ultrasound technique will be used for extraction process with ethanol-water as the solvent. Methanol and ethanol containing a small amount of acid are the most commonly used solvents (Mantell et al., 2002, 2004; Pinelo et al., 2005).

Solvent is needed to isolate anthocyanin from the mixture of stems and leaves. Ethanol-water is chose as solvent because it is suitable to use in food industry. Moreover ethanol-water has the capability to extract higher content of phenolic compound compare to other solvents. This can be proved by the study of (Ciencia, 2012) which found that the ethanol-water combinations resulted in a very high extraction of phenolic compounds, as high quantities of phenolic compounds were observed.

### *2.4 Parameters of the experiment*

To undergo the extraction process with ultrasound, several parameters will be adjusted to get the maximum yield of anthocyanin. The parameters involved are time, temperature and solvent ratio. The recovery of these components is commonly performed through a solvent-extraction procedure and the concentration of solvent, time, and temperature are important parameters to be optimized.(Spigno et al, 2007). These parameters will be observed to identify which condition produce the highest yield of anthocyanin. It is believed that all the parameters may affect the production of anthocyanin. The study of (cisse et al) showed that it has been found that the solid-to-solvent ratio and the particle size had a strong effect on both extraction velocity and anthocyanin extraction yield. The effect of temperature to the production of anthocyanin was found by (Chumsri et al.) which showed that it was found that greater extraction temperature and time contributed to less brillent red in color and also less in the amount of total anthocyanin contents. So it is needed in this study to observe these parameters so that a comparison of effective parameters which contribute to a higher production of anthocyanins can be decided and determined.

# 3 MATERIALS AND METHODS

## *3.1 Introduction*

This study main material was the mixture of stems and roots of *H.Sabdariffa* which come from Agriculture Department of Pahang. The chemicals involved in this study were Ethanol, Acetonitrile and Cyanidin Chloride. As for Cyanidin Chloride was purchased from Chemo Lab while other chemicals were obtained from University Malaysia Pahang chemical engineering laboratory. These chemicals that were used for this research are all analytical grade.

## *3.2 Materials*

i. *H.Sabdariffa* stems and roots

ii. Water

iii. Ethanol

iv. Acetonitrile

## *3.3 Apparatus*

i. Beaker

ii. Measuring cylinder

iii. Quartz tube

iv. Rotary Evaporator

v. Ultrasound

vi. High Performance Liquid Chromatography (HPLC)

vii. Grinder

viii. Vacuum filter

### *3.4 Overall Methodology Flow Chart.*

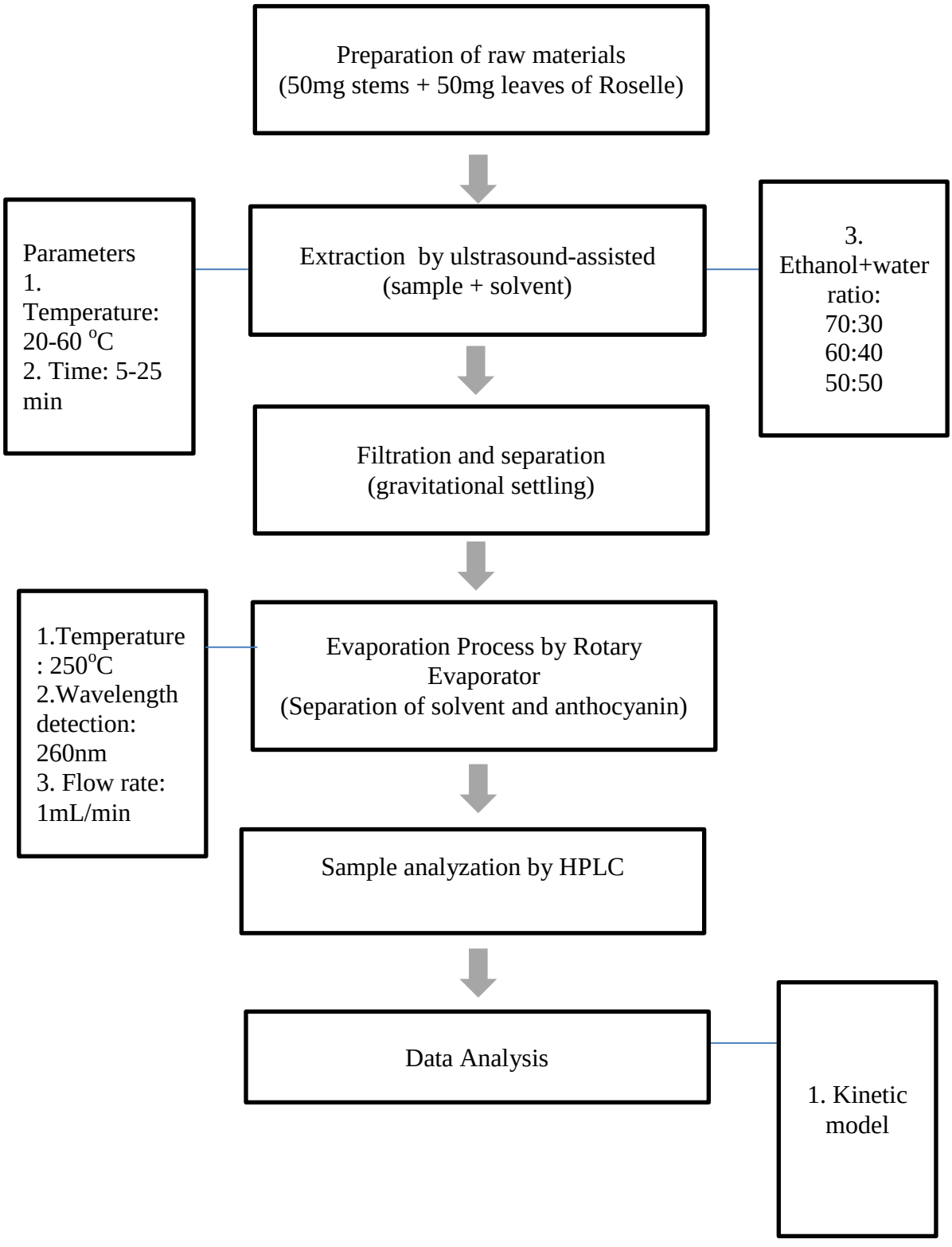


**Figure 3.1:** Overall Methodology Flow Chart.

## *3.5 Experimental Methodology*

### *3.5.1 Mixture of Stems and Leaves*

The mixture of *H.sabdariffa* stems and roots were obtained from Taman Pertanian Pahang and they were dried in an oven. After that they were grinded to powder using a grinder and sieved to make sure the total surface area was equal. Then the sample were stored at ambient temperature.

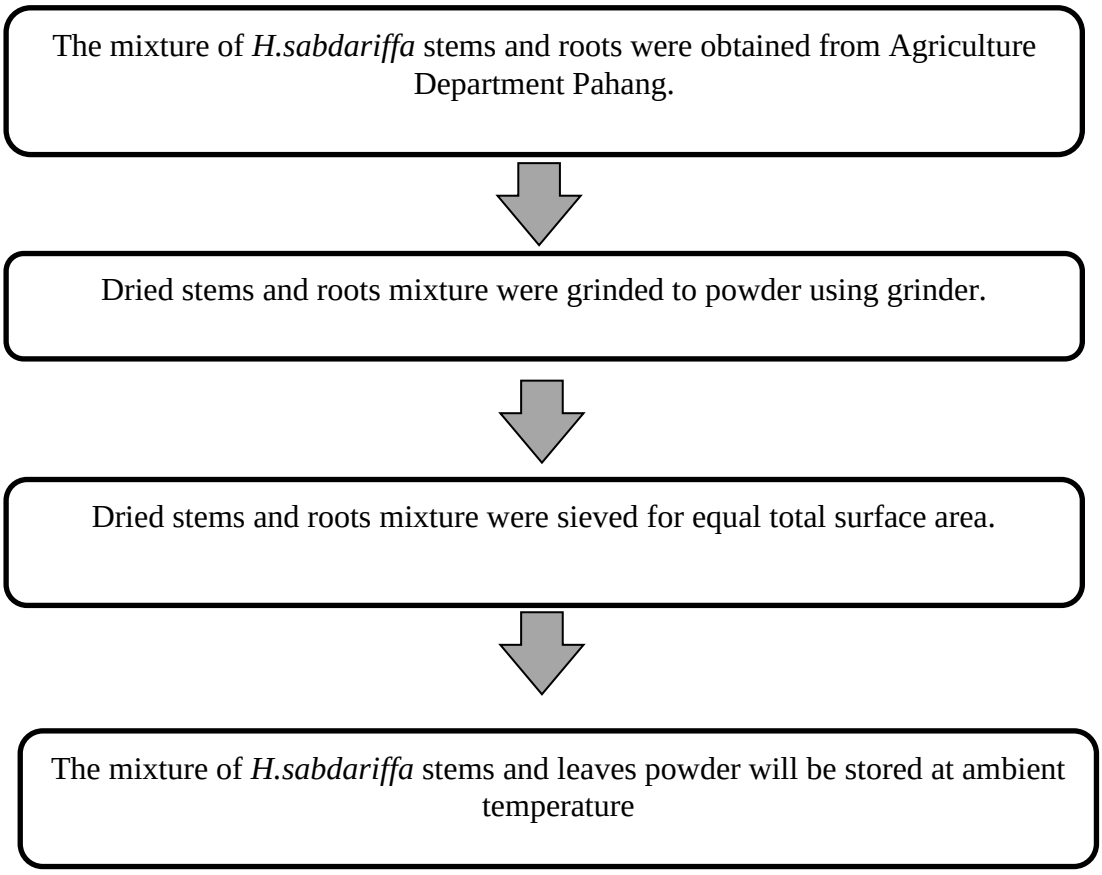


**Figure 3.2**: Flow diagram of preparation of *Hibiscus Sabdariffa* sample.

### *3.5.2 Ultrasound-Assisted Extraction Process*

Each 500mg sample of the mixture of *H.Sabdariffa* stems and roots powder were placed in a 50 ml quartz tube topped by a vapour condenser. The volume was made to 50 mL with the extraction solvent with different solvent ratio which ethanol as the solvent. Ultrasound-assisted extraction was performed in an ultrasonic bath. The parameters of time, temperature and solvent ration were adjusted. As for time, the range was between 10-40 min, the temperature is between 30-85$^{o}$C and solvent ratio of ethanol:water are 20:80, 40:60, 70:30 and 80:20. After extraction, the flask was immediately cooled to room temperature by using chilled water. The extract were filtered through filter paper and concentrated to dryness. Before proceed to the next process the sample were allowed to cool at room temperature.

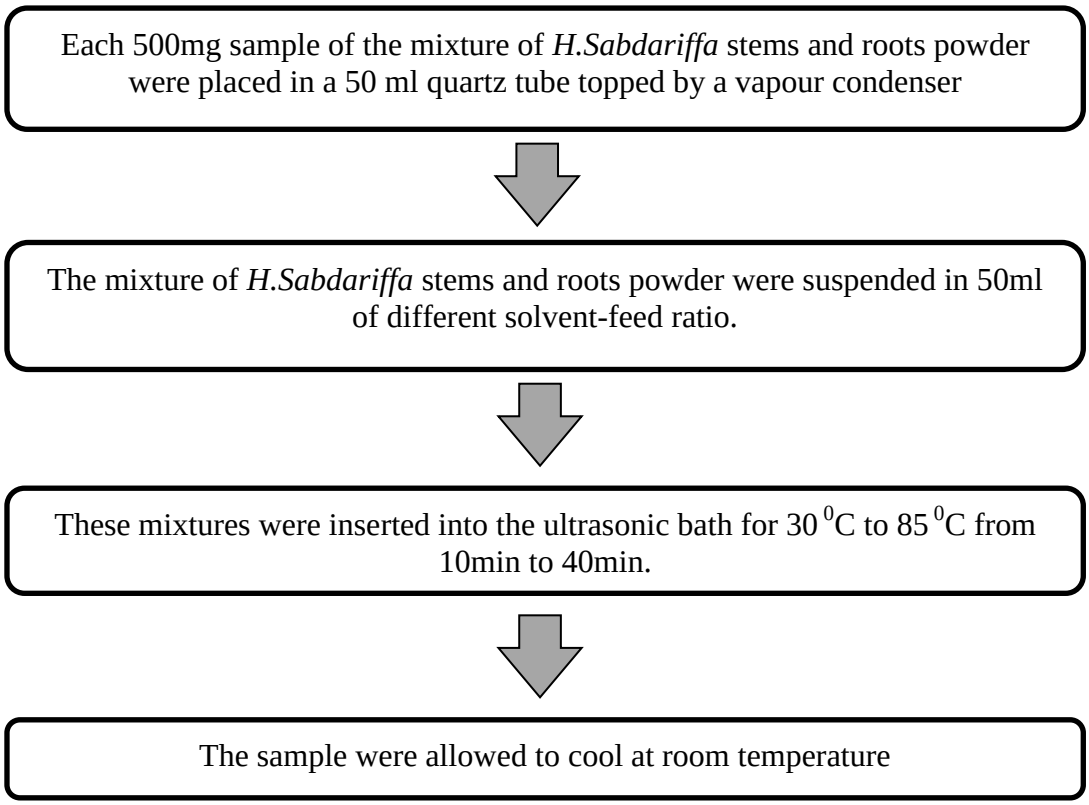


**Figure 3.3**: Flow diagram of extraction process using ultrasound-assisted extraction technique.

### *3.5.3 Filtration Process*

The filtration process for this study was done by vacuum filter. Firstly, the filter disk was dried in the oven at 103 $^{0}$C to 105$^{0}$C for 1 hour. Then was cooled in desiccators and weighed. After that Pipette 50 mL of water sample onto centre of filter disk in a Buchner flask, using gentle suction (under vacuum). Next the filtering apparatus were assembled and suction was begun. Lastly, filter was washed with three successive 10 mL volumes of distilled water, allowed complete drainage between washings, and continue suction for about 3 min after filtration was complete.

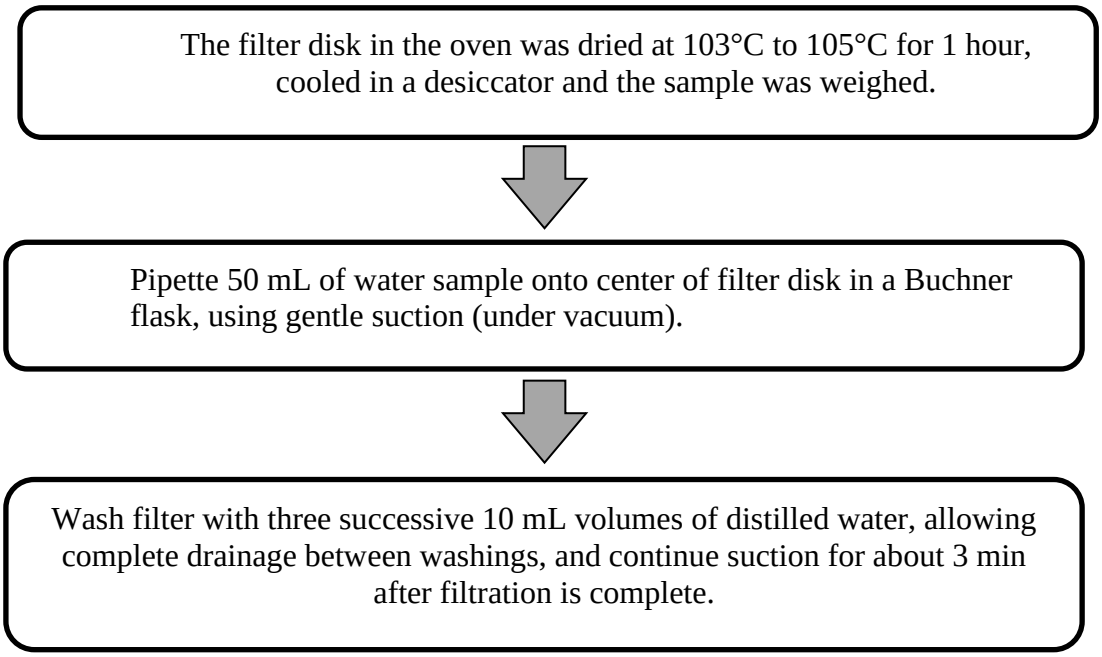


**Figure 3.4**: Flow diagram of filtration process using vacuum filter.

### *3.5.5 Evaporation Process*

Rotary evaporator was used to concentrate the Roselle extract. After the general start up procedures was performed, the temperature for extraction was set and let the temperature reached the set temperature. Sample was poured and rotated with suitable rotation speed and waited for the solvent to be evaporated and separated. Once there was no extracted solvent, the evaporation process was stopped and the volume was recorded.

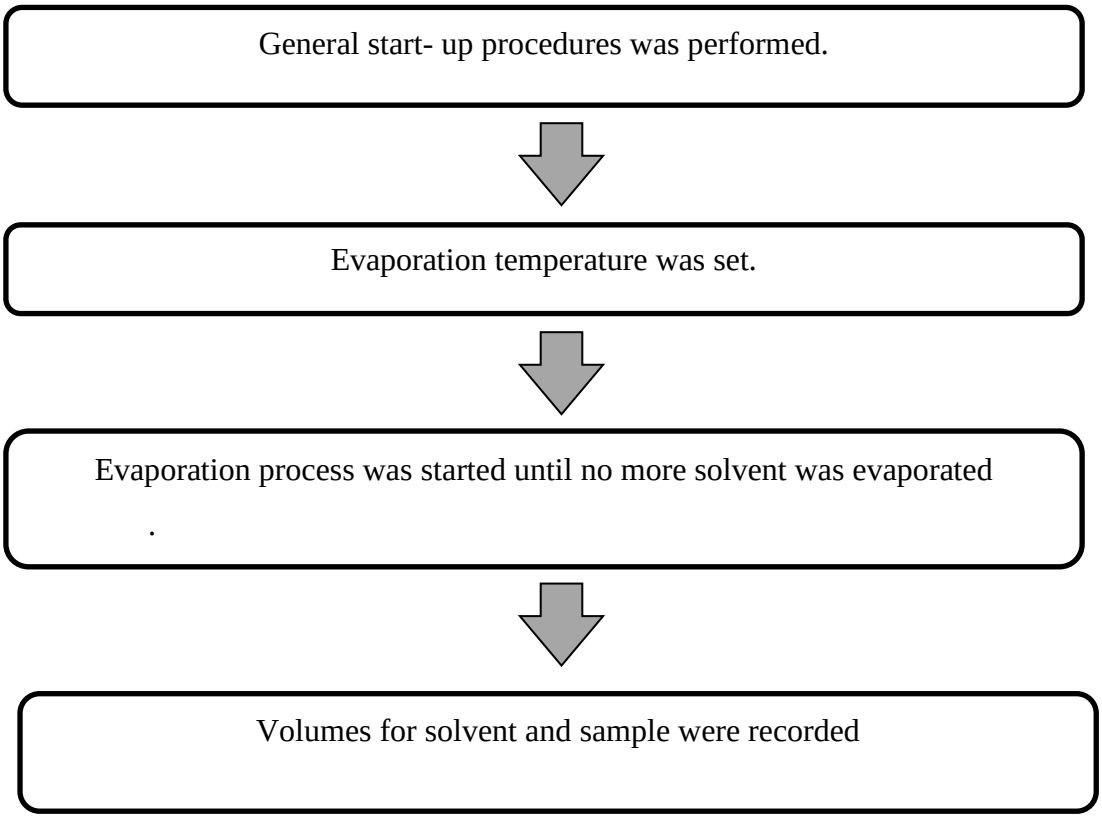


**Figure 3.5**: Flow diagram for evaporation process using rotary evaporator

### *3.5.6 Analyzation of sample*

The anthocyanins were quantified according to the Lee and Wrolstad (2004) method with modifications. Roselle extract were injected into the C-18 column and was eluted in an isocratic manner using acetonitrile:formic acid HPLC-grade solutions. The acetonitrile:formic acid mixture were applied at a flow rate of 1mL/min. The column temperature was 25$^{o}$C throughout the experiment. Then, the result was detected. Primary detection of was at 260 nm of wavelength.

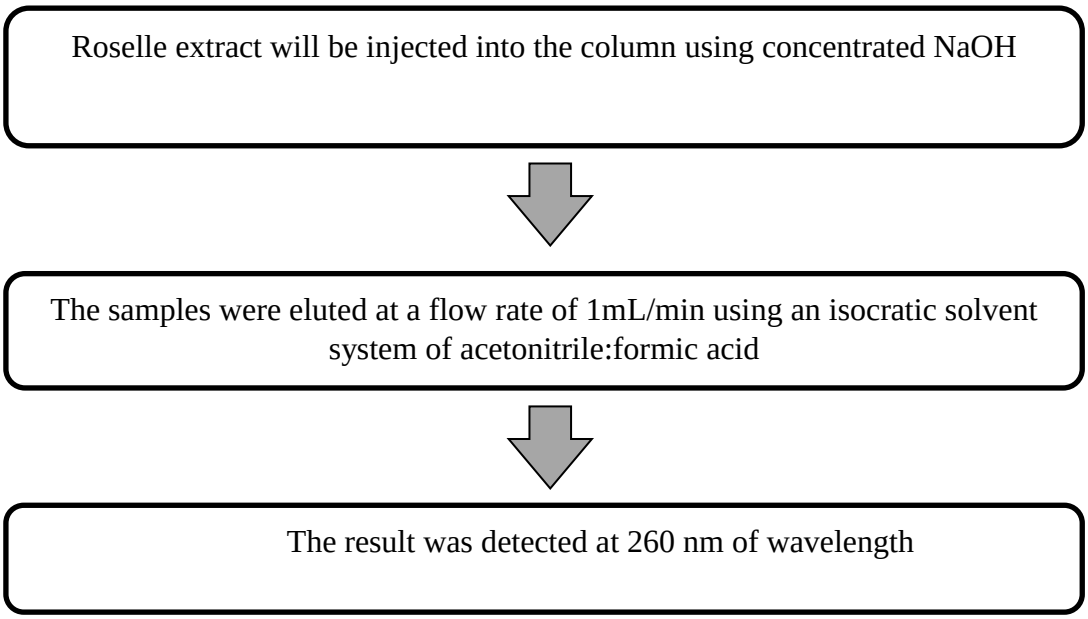


**Figure 3.6**: Flow diagram of the analysis using High Performance Chromatography (HPLC)

### *3.5.7 Analyzing Sample.*

#### *3.5.7.1 Preparation of Stock Solution*

1 mg of the standard solution were prepared by diluting with 50 % ethanol in water into 1 L volumetric flask respectively in prior to sonicate them for 15 minutes.

#### *3.5.7.2 Preparation of Standard Solution*

Standard solutions are prepared before running the High Performance Liquid Chromatography (HPLC). Solutions are prepared by diluting 1mg/mL of stock solution into a 10ml volumetric flask for concentration ranging from 0.02 to 0.1 ppm by using the equation.

$$M_1V_1 = M_2V_2$$